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Specific nitration of tyrosines 46 and 48 makes cytochrome *c* assemble a non-functional apoptosome

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ABSTRACT

Under nitroxidative stress, a minor fraction of cytochrome *c* can be modified by tyrosine nitration. Here we analyze the specific effect of nitration of tyrosines 46 and 48 on the dual role of cytochrome *c* in cell survival and cell death. Our findings reveal that nitration of these two solvent-exposed residues has a negligible effect on the rate of electron transfer from cytochrome *c* to cytochrome *c* oxidase, but impairs the ability of the heme protein to activate caspase-9 by assembling a non-functional apoptosome. It seems that cytochrome *c* nitration under cellular stress counteracts apoptosis in light of the small amount of modified protein. We conclude that other changes such as increased peroxidase activity prevail and allow the execution of apoptosis.

1. Introduction

Living cells obtain energy through oxidative phosphorylation, or mitochondrial respiration, which involves the transfer of electrons from NADH and $FADH_2$ to oxygen and the subsequent synthesis of ATP. The incomplete oxygen reduction leads to the formation of intermediate radicals, the so-called reactive nitrogen and/or oxygen species (RNOS) [1–3], which are usually eliminated by cellular detoxifying systems. Such mechanisms may fail during cell aging or under stress conditions, thereby increasing RNOS concentration.

One of the most deleterious reactive species is the strong oxidant peroxynitrite, which is formed by reaction between superoxide anion and nitric oxide. Peroxynitrite serves as an in vivo nitrating agent [4] that mainly promotes nitration of tyrosines in mitochondrial proteins [5–8], but its lifetime is long enough to cross the membrane and react with biomolecules in other compartments [5,6]. Actually, the cumulative protein tyrosine nitration might be responsible for alterations in protein function, turnover and localization, with the concomitant implication in the pathogenesis of diseases [9–13] undergoing nitroxidative stress.

Respiratory cytochrome *c* (C*c*) is one of the main targets for RNOS – and, in particular, for peroxynitrite – in mitochondria, where the heme protein is both nitrated and nitrosylated [14,15]. Under homeostasis, C*c* acts as an electron shuttle between the cytochrome bc_1 and cytochrome *c* oxidase (C*c*O) membrane-embedded complexes [16]. However, the pro-apoptotic stimuli make C*c* bind to and oxidize the mitochondria-specific phospholipid cardiolipin (CL) [17], which in turn allows the translocation of C*c* into the cytoplasm so as to trigger the apoptosis signalling pathway upon binding to the apoptosis protease-activation factor (Apaf-1) and apoptosome assembly [18,19]. In vitro nitration of human C*c* tyrosine residues at positions 67, 74 and 97 impairs the two antagonist functions of C*c* in cell life (respiration) and cell death (apoptosis) [20,21], in agreement with previous data obtained in vivo with Tyr67-nitrated C*c* [22]. In contrast, tyrosine nitration can increase the peroxidase activity of C*c* [21,23,24], an example of gain-of-function

Abbreviations: Ac-LEHD-AFC, N-acetyl-Leu-Glu-His-Asp-(7-amino-4-trifluoromethyl coumarin); Apaf-1, apoptosis protease-activating factor-1; CL, cardiolipin; C*c*, cytochrome *c*; C*c*O, cytochrome *c* oxidase; PC9, pro-caspase 9; RNOS, reactive nitrogen/oxygen species

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modification that sheds light to the biological significance of nitration since a small fraction of nitrated Cc may be sufficient to elicit a substantive biological signal.

Most physicochemical and functional studies of Cc have been performed with the horse protein modified by tyrosine chemical nitration at positions 67, 74 and 97 [20–27]. Significant differences are observed depending on whichever the nitrated residue is. Actually, previous reports describe how nitration of Tyr74 modulates the Cc functions, whereas nitration of Tyr97 has no any functional effect [20,21].

In the case of Tyr46 and Tyr48 of human Cc, which are solvent-exposed and easily nitrated in vitro [28], the mechanism by which the $-NO_2$ radical alters the Cc functions remains unclear. Here we have designed two human Cc mutants with all but one of their tyrosine residues – at position 46 or 48 – replaced by phenylalanines. Our experimental data demonstrate that in vitro nitration of either Tyr48 (which is a highly conserved residue in all organisms) or Tyr46 (which is only present in human and plant Cc) leads to the assembly of a non-functional apoptosome, which fails in caspases activation.

2. Materials and methods

2.1. Sample preparation

Recombinant human respiratory Cc, either the WT species or the monotyrosine mutants in which only Tyr46 or Tyr48 is present (the herein called *h*-Y46 or *h*-Y48 variants), were expressed in *Escherichia coli* DH5α strain and further purified by ionic exchange chromatography, as previously described [20,21]. Peroxynitrite synthesis and nitration of monotyrosine Cc mutants were performed as previously described [20,21,28] with the following modifications: Fe^{3+}-EDTA concentration and the number of peroxynitrite additions were increased up to 1.5 mM and 10 bolus additions, respectively. The nitration reaction was performed under acidic conditions (pH 5.0).

The nitrated Cc species were intensively washed in 10 mM potassium phosphate at pH 6 and purified to 95% homogeneity, as reported in Ref. [20]. Purity of nitrated Cc preparations, as well as molecular mass and specific nitrated tyrosine of each mutant, were confirmed by tryptic digestion and MALDI-TOF (Bruker-Daltonics, Germany) analyses. Western Blotting Solution (Amersham) with antibodies anti-nitrotyrosine (Biotem) was used to confirm the presence of $-NO_2$ groups in the Cc samples upon nitration. Samples were concentrated to 0.2–2.0 mM in 5 mM sodium phosphate buffer (pH 6). The pyridine hemochrome assay was used to estimate the extinction coefficients of the nitrated and non-nitrated forms of monotyrosine mutants [29]. When oxidation of methionine residues was detected, the samples were discarded.

Recombinant human Apaf-1 was expressed and purified as described in Refs. [30,31]. Recombinant pro-caspase 9 (PC9) was produced and purified as in Ref. [21]. Horse cytochrome *c* oxidase (CcO) was purified as reported in Ref. [20]. CcO concentration was estimated by using a differential extinction coefficient $\Delta\varepsilon_{604-630}$ of 17 mM^{-1} cm^{-1} for the reduced *minus* oxidized protein [32].

2.2. Kinetic analysis

The kinetics of electron transfer from the non-nitrated and nitrated Cc species to horse CcO were analyzed by laser flash spectroscopy by following the absorbance change at 550 nm. The redox reactions were induced by EDTA-photoreduced FMN, as previously reported [20]. All experiments were performed under pseudo-first order conditions, with the concentration of oxidized CcO well exceeding that of reduced Cc per flash. Further kinetic analyses were carried out to estimate the bimolecular rate constant (k_2) for the nitrated and non-nitrated mutants, as well as the association (K_A) and effective electron transfer rate (k'_{et}) constants for WT Cc [20].

2.3. Apaf-1/Cc cross-linking, light scattering and caspase-9 activation

To detect the interaction between Cc and Apaf-1 in *Jurkat* T cell extracts, the cross-linking, light scattering and caspase-9 assays were run as described in Ref. [21].

3. Results

3.1. Nitration of monotyrosine mutants of Cc

Nitrated monotyrosine Cc mutants in which only Tyr46 or Tyr48 is present were separated from non-nitrated protein in a CM-cellulose column equilibrated with 1.5 mM borate, pH 9.0, using a 0–100 mM NaCl gradient. Nitrated Cc eluted at a much lower salt concentration than native protein because of the extra negative charge of deprotonated tyrosyl anions, whose pK_a is modified by the strong electron-withdrawing effect of the substituent $-NO_2$ group at the 3-position [24]. The purity to homogeneity of nitrated Cc preparations was corroborated by SDS–PAGE and Western Blot using antibodies anti-nitrotyrosine (Biotem) to detect the presence of the $-NO_2$ group (Fig. 1). In addition, the molecular mass and the specifically nitrated tyrosine residue of each mutant were confirmed by tryptic digestion and MALDI-TOF (Bruker-Daltonics, Germany) analyses, as recently reported [28].

3.2. Electron transfer between oxidized Cc mutants and CcO

Cc serves as a one-electron carrier between cytochrome bc_1 complex and CcO at the end of the mitochondrial electron transport chain. In a previous report [20], the kinetics of horse CcO reduction by the nitrated and non-nitrated species of the *h*-Y67, *h*-Y74 and *h*-Y97 mutants were studied by laser flash spectroscopy. Here, we have analyzed the effect of nitration of Cc at positions 46 and 48 using the *h*-Y46 and *h*-Y48 variants to reduce CcO. As can be seen in Fig. 2, WT Cc shows a non-linear dependence of the observed pseudo-first-order rate constant (k_{obs}) upon CcO concentration at pH 6.5, thus indicating the formation of a kinetically detectable transient Cc–CcO electron transfer complex, as previously observed at pH 7.5 [20]. However, the k_{obs} values at pH 6.5 with the non-nitrated and nitrated forms of *h*-Y46 and *h*-Y48 show in all cases a linear dependence on CcO concentration. This suggests that electron transfer is much faster than complex dissociation, in agreement with a collisional reaction mechanism [33]. The resulting values for the bimolecular rate constant (k_2) estimated with the two Cc mutants show that nitration slightly decreases the ability of *h*-Y46 to donate electrons to CcO and has an even lower effect on *h*-Y48 (Table 1). At pH 7.5, the effect of nitration on the k_2 values with *h*-Y46 and *h*-Y48 is practically negligible (not-shown).

3.3. Cc-dependent activation of caspases

To check how the nitration of Cc alters the apoptotic process, the apoptosome was first reconstituted in vitro by incubating recombinant Apaf-1 with either the nitrated or non-nitrated species of *h*-Y46 and *h*-Y48. The subsequent addition of PC9 allowed to follow its activation to caspase-9 by fluorometric methods.

The cross-linking and light-scattering assays demonstrated that Cc binds to Apaf-1 independently of whichever tyrosine residue – Tyr46 or Tyr48 – is modified (Fig. 3). In fact, the light scattering of Apaf-1 increases upon addition of any of the Cc mutants (Fig. 3B).

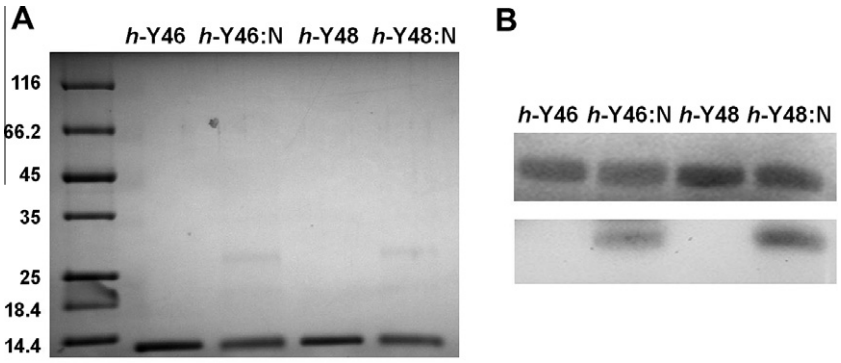

Fig. 1. Determination of purity of nitrated C*c* samples. (A) SDS–PAGE electrophoresis of non-nitrated and nitrated *h*-Y46 and *h*-Y48 mutants. Each protein (1.3 μg) was loaded onto a 12% SDS–PAGE gel. (B) Immunodetection of nitrated C*c* mutants by Western blot analysis using anti Tyr-NO_2 antibodies. *Upper* – Transfer of non-nitrated and nitrated *h*-Y46 and *h*-Y48 samples to the nitrocellulose membrane as corroborated by Ponceau S solution staining. *Lower* – Detection of Tyr-NO_2 just in the *h*-Y46:N and *h*-Y48:N samples previously submitted to the nitration protocol.

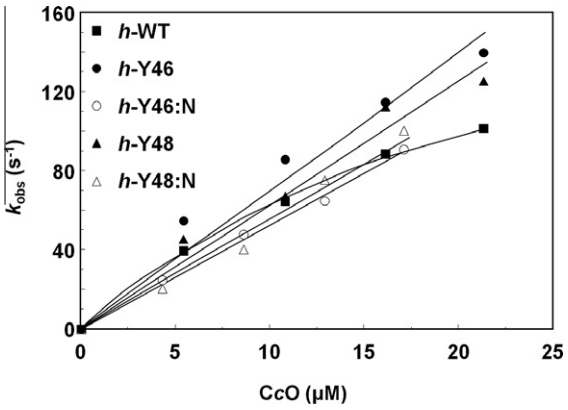

Fig. 2. Dependence of k_{obs} for oxidation of *h*-Y46 and *h*-Y48, in their non-nitrated or nitrated forms, upon C*c*O concentration. The reaction mixture contained (in a final volume of 0.8 mL) 10 mM Tris–HCl, pH 6.5, 2 mM EDTA, 0.07% dodecyl β-maltoside, 50 mM KCl, 100 μM FMN, and 40 μM C*c*. All the experiments were performed at room temperature. Other experimental conditions were as described under Section 2. The resulting kinetic parameters are summarized in Table 1.

Table 1
Kinetic parameters for oxidation by horse C*c*O of different human C*c* species at pH 6.5.

C*c* species	$k_2 \times 10^{-6}$ ($M^{-1}\ s^{-1}$)	$K_A \times 10^{-4}$ (M^{-1})[a]	k'_{et} (s^{-1})[a]
h-WT	–	3.7	232
h-Y46	7.0	–	–
h-Y46:N	5.2	–	–
h-Y48	6.3	–	–
h-Y48:N	5.6	–	–

[a] K_A and k'_{et} values were estimated as in Ref. [20].

The inability of the two nitrated C*c* variants to activate caspase-9 (Fig. 4) suggests that they yield a non-functional apoptosome. The tyrosine-by-phenylalanine mutations themselves do not alter the ability of C*c* to activate caspase-9 at pH 7.5. Therefore, these two tyrosine residues are not essential for triggering apoptosis in vitro (Fig. 4). In contrast, nitration at positions 46 or 48 fully inhibits caspase-9 activation. At the highest C*c* concentration, only *h*-Y46:N retains 20% of its ability to activate caspase-9, as was shown for the *h*-Y74:N species [21]. Therefore, the inhibitory effect on caspase-9 activation observed with the polynitrated *h*-WT species could be ascribed to the specific nitration of Tyr46 and/or Tyr48 rather than to any cooperative effect among different Tyr-NO_2 groups [20].

4. Discussion

Cc is involved in two opposite biological functions: cell life (mitochondrial respiration) and cell death (apoptosis), which are regulated by post-translational modifications such as nitration [20,34]. Actually, the addition of a $-NO_2$ group to any tyrosine residue yields different effects depending on the position of the modified residue at the heme protein structure.

In this context, it is worth noting that C*c* becomes a high-spin species upon Tyr46 or Tyr48 nitration at physiological pH although the overall folding remains unaltered [28], a finding that may explain the drop of ca. 100 mV in the midpoint redox potential value of the nitrated C*c* species [20]. Such a drop in redox potential may roughly disrupt the cellular respiration. As inferred from the k_2 values for C*c*O reduction by non-nitrated and nitrated proteins at physiological pH, nitration at positions 46 and 48 barely affects the C*c* reactivity. However, C*c* nitrated at these two positions is no longer isopotential with cytochrome c_1 and may thus be unable to accept electrons from the cytochrome bc_1 complex. Actually, the excess in RNOS yielded from the first complexes of the respiratory chain under nitroxidative stress could lead to a positive nitration-driven feedback cycle, with cytochrome bc_1 promoting the increase in RNOS and nitrated C*c*.

Upon leaving the mitochondria under nitroxidative stress, C*c* nitrated at positions 46 and 48 could inhibit the apoptosis signal by forming a non-functional apoptosome. However, the binding affinity of nitrated C*c* species towards Apaf-1 is substantially lower than those of native C*c* (data not shown). Nitration of Tyr46 and Tyr48 rearranges the H-bond network and turns the alkaline transition into a physiologically relevant process [32]. Actually, the alkaline transition pK_a is shifted towards neutral pH values, with the concomitant replacement of Met80 by Lys73 or Lys79 as heme axial ligand. As a consequence, the alkaline form of nitrated C*c* is predominant at pH 7.5, which is the optimal pH value for caspase activation. In the low-spin, alkaline structure of C*c*, the Ω-loop – which has previously been reported to be key for the interaction with Apaf-1 through residues Lys72 and/or Lys73 [20,35,36] – undergoes a substantial conformational change. This explains the assembly of a non-functional apoptosome, which would be unable to activate caspases and to drive cells to apoptosis.

It has recently been reported that tyrosine 48 gets phosphorylated under homeostatic conditions [37,38], with the concomitant effect on the availability of C*c* to activate caspases [39,40]. Nitration and phosphorylation of C*c* at the same tyrosine residue are mutually exclusive [41] but inhibit C*c*-dependent caspases activation with a similar efficiency. In the case of Tyr48 phosphorylation, the electron transfer is also inhibited [39]. In summary, Tyr48

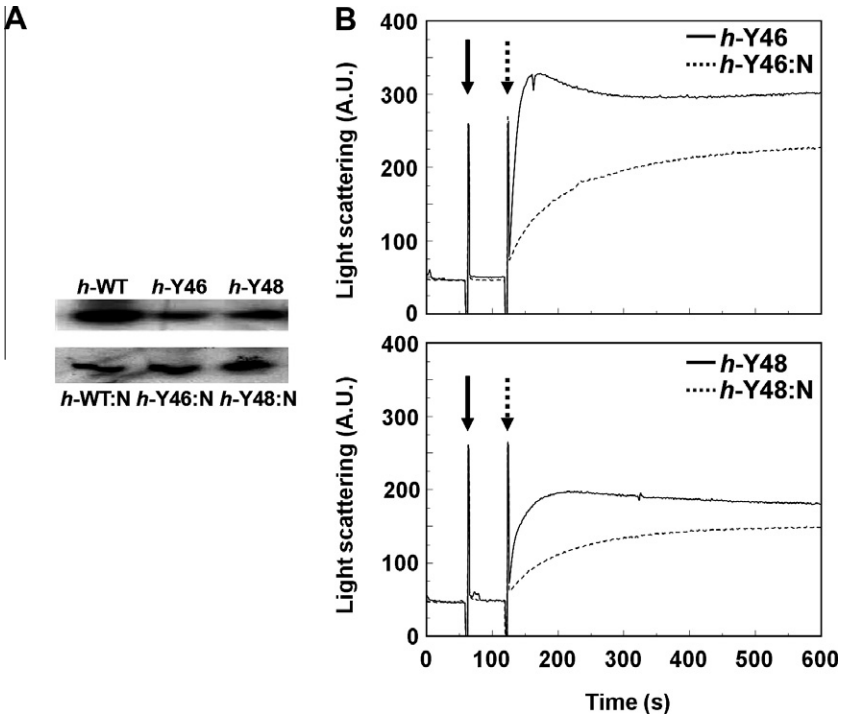

Fig. 3. Interaction between Apaf-1 and oxidized C*c* variants. (A) Cross-linking between Apaf-1 and the non-nitrated or nitrated *h*-Y46 and *h*-Y48 mutants, as detected by Western blot using antibodies against C*c*. (B) Formation of the complex between Apaf-1 and the non-nitrated (continuous line) or nitrated (dashed line) C*c* mutants, as determined by light scattering. The arrows stand for addition of Apaf-1 (solid line) and C*c* (dashed line) to the buffer solution.

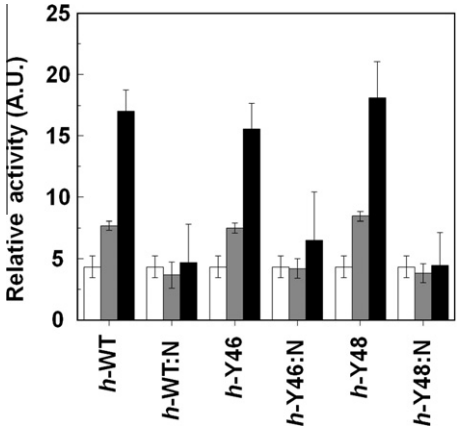

Fig. 4. C*c*-dependent activation of caspase-9 upon interaction of Apaf-1 with oxidized C*c* mutants. Caspase activation was determined by following the increase in fluorescence after substrate (Ac-LEHD-AFC) cleavage subsequent to incubation of Apaf-1 and PC9 with nitrated or non-nitrated C*c* mutant. C*c* concentrations were: 0 (white), 20 nM (grey) and 40 nM (black).

phosphorylation under homeostasis [37,38] and Tyr48 nitration under oxidative stress may act as anti-apoptotic switches that make C*c* fail in assembling a functional apoptosome.

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